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Novel Applications of Fluorescence Sensors

KENNETH F. REARDON,*[,1] THOMAS SCHEPER,[2] KLAUS-DIETER ANDERS,[2] WOLFGANG MÜLLER,[2] AND A. FRITZ BÜCKMANN[3]

[1]Department of Agricultural and Chemical Engineering, Colorado State University, Fort Collins, CO 80523 USA; [2]Institut für Technische Chemie, Universität Hannover, 3000 Hannover 1 FRG; and [3]GBF-Braunschweig, 3300 Braunschweig FRG

ABSTRACT

Typically, NAD(P)H-sensitive culture probes have been used to estimate biomass concentrations in suspended-cell cultivations, but these sensors have other uses as well. A number of applications, ranging from biosensors to immobilized-cell metabolic studies, are presented.

Index Entries: Fluorescence; sensor; NADH; metabolism; immobilized cells.

INTRODUCTION

In the last several years, there have been an increasing number of reports on the uses of miniaturized fluorescence probes in biotechnology. These probes were designed to measure the fluorescence of the reduced forms of intracellular nicotine adenine dinucleotides (NADH and NADPH) in bioreactors. The large majority of these applications have utilized the on-line, *in situ* NAD(P)H-dependent culture fluorescence measurements obtained from these sensors to estimate suspended-cell biomass concentrations (e.g., *1–3*); some have also involved studies of suspended-cell metabolism.

In this report, several new applications of fluorescence sensors are presented:

*Author to whom all correspondence and reprint requests should be addressed.

1. As the optrode in an optical biosensor system, monitoring cofactor-dependent enzymatic reactions;
2. As the detector in a "toxin guard" system, in which intracellular NAD(P)H concentration changes are monitored to detect cell-toxic compounds in a feed stream; and
3. In studies of immobilized-cell systems, with specific examples concerning the effects of diffusion limitation in alginate beads, glucose pulses on immobilized yeast, and metabolic shifts and nutrient feeding of immobilized *Clostridium acetobutylicum*.

The goal of this report is to convey the range of uses that fluorescence probes have in biotechnology. To this end, each experimental set-up and some associated results are discussed relatively briefly. Further details on particular applications can be obtained from the appropriate references.

MATERIALS AND METHODS

All of the experiments utilized the Ingold Fluorosensor, one of two commercially available fluorescence probes. The usefulness of these probes stems from the fact that NADH and NADPH fluoresce (maximum excitation wavelength 340 nm, maximum emission wavelength 460 nm), but their oxidized forms (NAD^+ and $NADP^+$) do not. The Fluorosensor employs a low-pressure mercury lamp as a light source; light from this lamp is passed through interference filters to produce a 360-nm light beam. The ultraviolet light is then guided through the probe tip and into the surrounding fluid by a quartz fiber optic bundle. Fluorescent light (460 nm) is collected at the optical head in the probe tip and guided by a second fiber optic bundle to a photomultiplier, from which the signal is transferred to an external processing unit.

Data from fluorescence probes are typically expressed as "relative fluorescence" (in volts) because the important quantities are the magnitudes of increases or decreases in the measured fluorescence, not absolute values. The use of relative fluorescence also allows one to subtract constant background fluorescence levels.

Details of the experimental set-up for each application are given in the associated discussion and references, as is information on cells and cultivation conditions.

RESULTS AND DISCUSSION

The Fluorescence Probe as a Biosensor Optrode

An optical biosensor system for the measurement of lactate or pyruvate has been developed using the Fluorosensor as the optrode (4). (The complete system is illustrated in Fig. 1.) The tip of the fluorescence probe

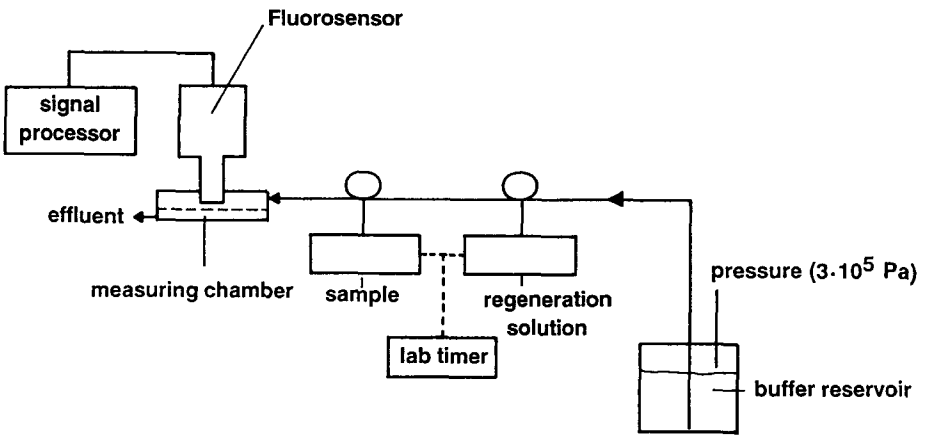

Fig. 1. Optical biosensor system.

fitted into a measuring chamber that had a volume of 1 mL. This measuring chamber contained a specially designed magnetic stirrer and an ultrafiltration membrane (5 kDalton cutoff). A carrier stream of 50 m*M* potassium phosphate buffer (pH 8.5) flowed continuously through the chamber at 0.18 mL/min. The entire system was operated under pressure (3.5×10^5 Pa) to ensure good flow through the ultrafiltration membrane.

Before the analysis of lactate-containing samples, the chamber was loaded with macromolecular polyethylene glycol-NAD^+ (20,000 molecular weight) (*5*) and two enzymes, lactate dehydrogenase (LDH) and alanine transaminase (glutamic-pyruvic transaminase), which catalyze, respectively, the reactions:

$$\text{lactate} + NAD^+ \rightleftharpoons \text{pyruvate} + NADH + H^+$$

$$\text{pyruvate} + \text{L-glutamate} \rightarrow \text{L-alanine} + \alpha\text{-ketoglutarate}$$

The enzymes and the high molecular weight NAD^+ were retained in the measuring chamber by the membrane, but smaller molecules were able to pass through.

Lactate concentration measurement began with the injection of sample into the buffer flow. In the chamber (Fig. 2), the lactate was converted to pyruvate by LDH. The resulting increase in PEG-NADH fluorescence was proportional to the lactate concentration. The transamination reaction was required to shift an otherwise unfavorable equilibrium, and it was necessary to add glutamate (a substrate in the second reaction) to the sample solution.

After a measurement, the PEG-NADH had to be reoxidized to PEG-NAD^+; this was done by automatically injecting a pyruvate solution after each analysis and operating the LDH reaction in reverse.

Pyruvate could be measured by monitoring the fluorescence decrease caused by the reverse of the LDH reaction. In this case, glutamate was not

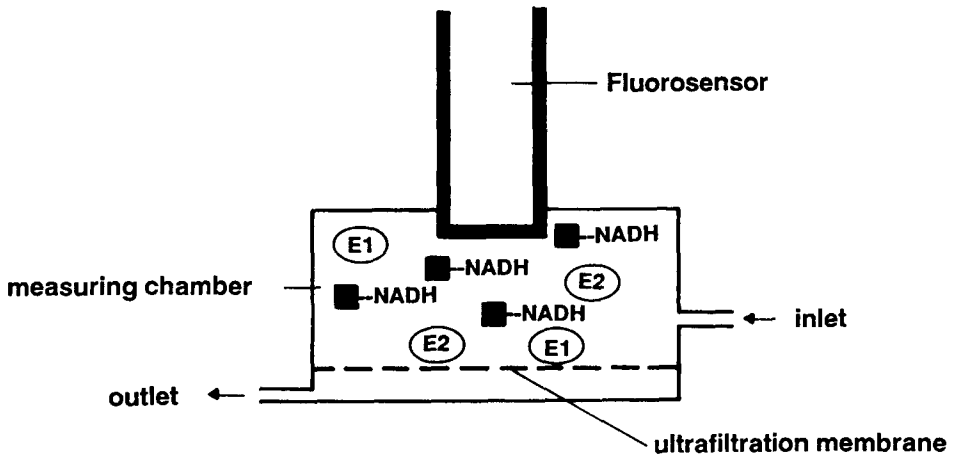

Fig. 2. Principle of analysis: E1 and E2 are the enzymes used in the assay, lactate dehydrogenase and alanine transaminase. ■-NADH represents high molecular weight PEG-NADH.

added to the sample. For pyruvate assays, the cofactor (PEG-NADH) was regenerated by the addition of a solution containing lactate and glutamate.

Figure 3 shows a sample fluorescence trace from several lactate tests. The fluorescence signal increased rapidly after each sample injection (noted by the "C" values) and reached a plateau shortly thereafter. The addition of the pyruvate (regeneration) solution at the times marked "R" caused the signal to decrease, and the sensor was soon ready for another test sample. The response of the system was quite consistent (note the two injections of concentration C_1). Figures 4A and 4B present the calibration curves for the lactate and pyruvate assays, respectively, in which the fluorescence increase or decrease correlates well with the analyte concentration.

The system was relatively stable and the half-lives of the enzymes were about 60 h at 30°C.

The Use of a Fluorescence Probe in a "Toxin Guard" System

The concept of a toxin guard system stems from the need to protect the microbial communities in activated sludge reactors in wastewater treatment facilities from toxic levels of chemicals that occasionally enter the feed (sewage) stream. The same idea could be extended to other situations in which it is necessary to guard against contaminated inlet flows. A system for this purpose is shown in Fig. 5. Here, the NAD(P)H-dependent fluorescence of calcium alginate-immobilized *Saccharomyces cerevisiae* H1022 was monitored. The metal column containing the immobilized cells was connected to the fermentor via a recirculation loop, and medium (defined, with 3% glucose) was pumped through the alginate-bead bed at high

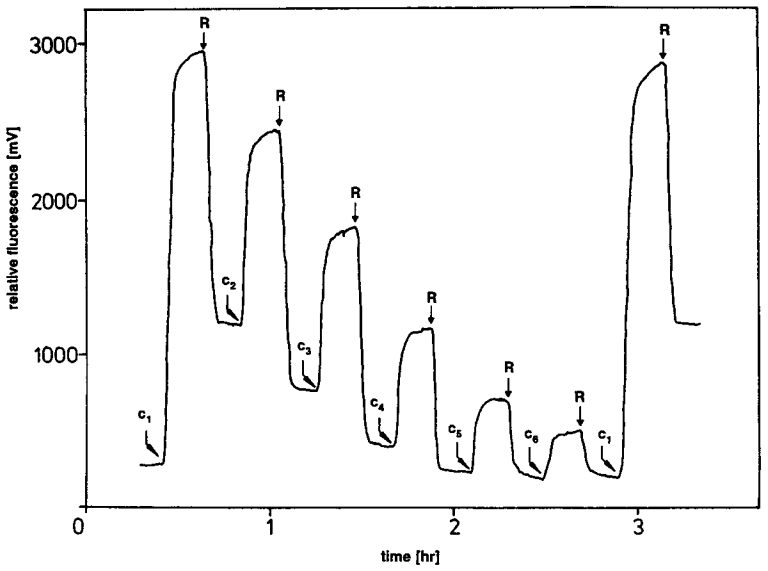

Fig. 3. Examples of lactate analysis using the optical biosensor (C_1=5 g/L, C_2=2.5 g/L, C_3=1.25 g/L, C_4=0.625 g/L, C_5=0.313 g/L, C_6=0.156 g/L). The samples were injected at the times marked by the "C" labels; "R" marks the times of cofactor regeneration solution addition.

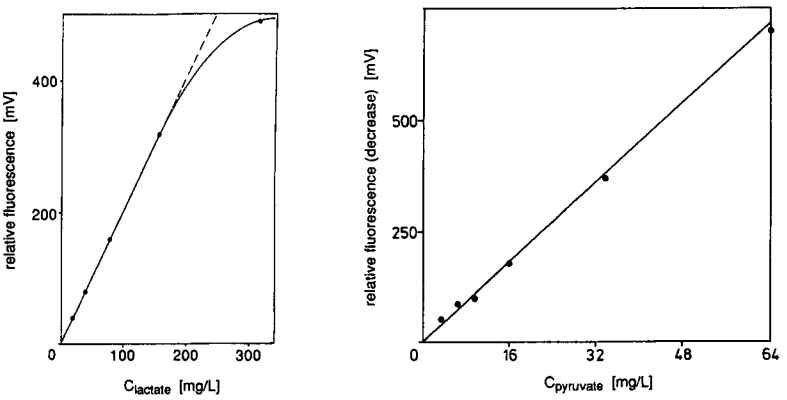

Fig. 4. A) Lactate calibration curve; B) pyruvate calibration curve.

flow rates (340 mL/min); the total liquid volume was 1.2 L. The fermentor was used to provide aeration, stirring, temperature (30°C), and pH control (pH 4). The Fluorosensor was attached to the column at a quartz window. Further experimental details are available elsewhere (*6*).

Figure 6 shows the result of an experiment in which 1 mL of 0.3 m*M* 2,4-dinitrophenol (DNP), an uncoupling agent, was injected into the

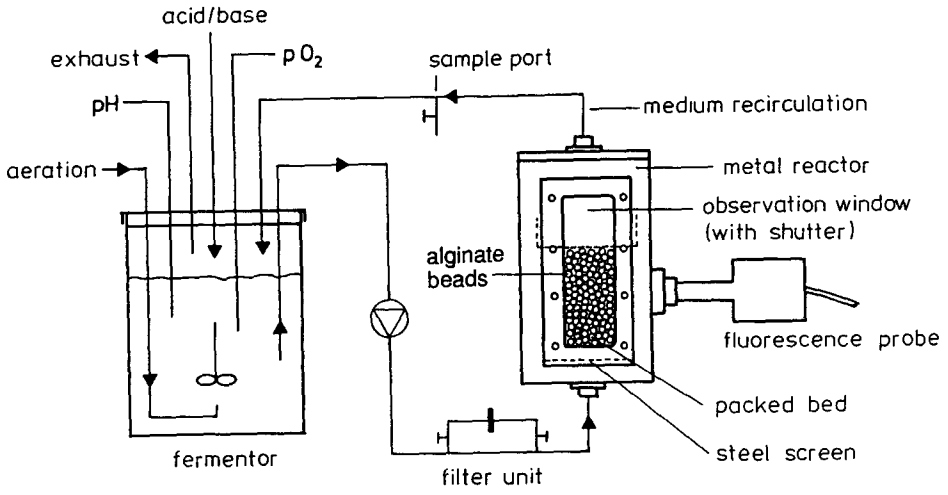

Fig. 5. Immobilized-cell reactor system.

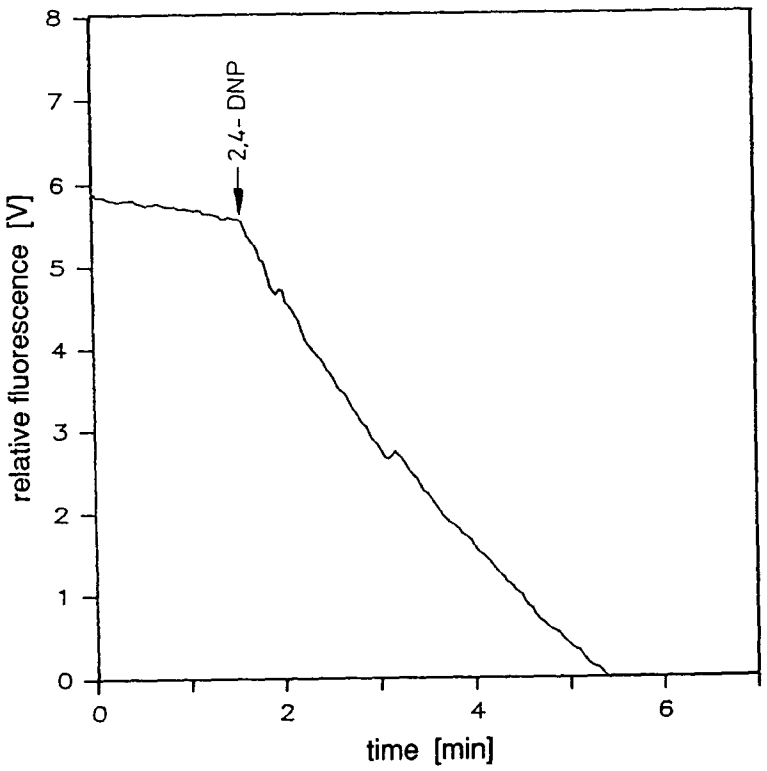

Fig. 6. Fluorescence profile during the toxin-pulse experiment. The arrow marks the injection of 2,4-dinitrophenol.

medium. Uncouplers prevent the conversion of ADP to ATP but do not stop electron transport. The effect of the blockage of ADP phosphorylation is the uncontrolled oxidation of NADH, measured as a rapid decrease in the fluoresence signal.

The rapid, sensitive response of the immobilized yeast intracellular NADH pool to this toxin suggests the use of this immobilized cell/fluorescence probe system to monitor fermentor feed streams for the presence of toxins as well as for changes in medium composition.

The Use of Fluorescence Probes in Studies of Immobilized-Cell Systems

A number of publications have demonstrated the usefulness of fluorescence sensors in studies of suspended-cell metabolic phenomena (e.g., anaerobic–aerobic transitions) (*1–3,7,8*). It is well known that the redox state of the cell changes rapidly in response to environmental changes; in some cases, metabolic shifts can also cause changes in the NADH/NAD^+ ratio. The measurement of NAD(P)H-dependent fluorescence can provide insights into these events that cannot easily be obtained from other techniques.

It is also possible to use fluorescence probes to study immobilized-cell cultivations on-line. Several examples of both types of investigation will be presented briefly.

Diffusion Limitation of Entrapped Cells

It is well known that entrapment techniques for cell immobilization frequently result in diffusion-limited cultivations. However, these effects are often difficult to observe experimentally. In order to determine if the effects of mass-transfer limitation could be detected with a fluorescence probe, several batch cultivations were performed using calcium alginate-mmobilized *S. cerevisiae* growing on a synthetic glucose medium. These experiments were carried out in the reactor system shown in Fig. 5. In each experiment, a different alginate bead diameter was used (1.4, 2.2, and 3.0 mm) and in one experiment, 3.0-mm-diameter beads were coated with a 0.2-mm cell-free alginate layer (*9*).

The fluorescence profiles from these experiments are shown in Fig. 7, where they have been offset from one another to better show their features. The first peak in each profile, marked by the dotted line, is typical in batch fermentations on glucose medium and occurs at the start of the diauxic lag phase (between glucose depletion and the start of ethanol consumption). It is clear that the increased diffusion limitation caused by the larger bead diameters affected the size and timing of this peak as well as other features in the fluorescence curves.

Glucose Pulses and Immobilized Yeast

A different type of immobilized-cell reactor was used for short-term investigations on the effects of glucose pulses (*9*). This small reactor (Fig. 8)

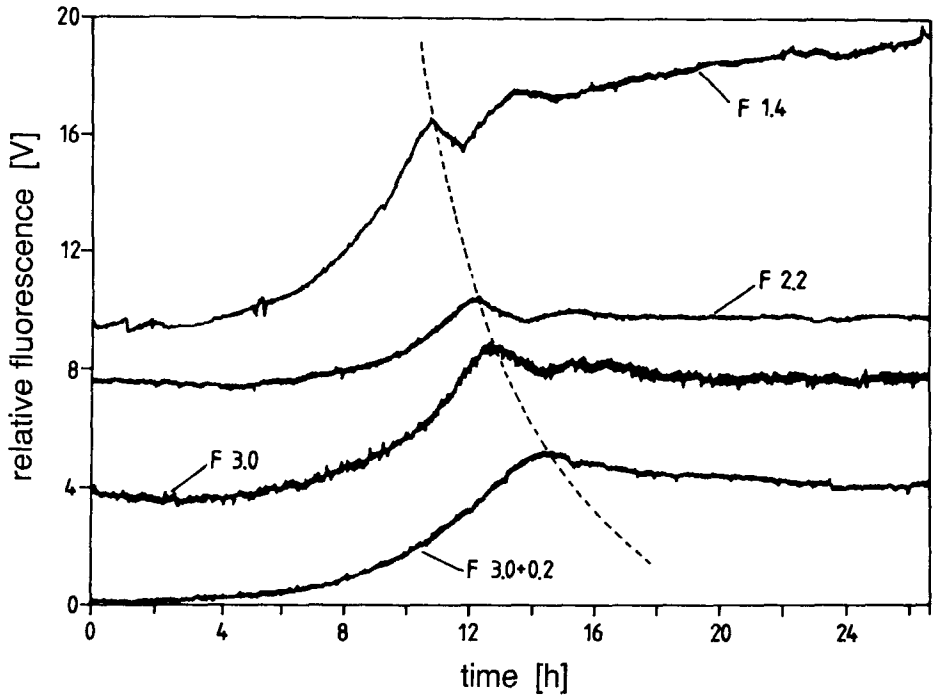

Fig. 7. Immobilized *S. cerevisiae* batch growth fluorescence curves. The F value indicates the bead diameter in mm ("F 3.0+0.2" curve is for 3-mm beads with a 0.2-mm cell-free alginate outer layer). The dotted line indicates the peaks that occurred at the diauxic lag phase.

fitted over the probe tip of the Fluorosensor; thin layers of calcium alginate-immobilized *S. cerevisiae* on glass slides were then fixed in front of the probe. Oxygenated, glucose-free defined medium was pumped continuously through the chamber above the cells, and various samples could be injected into this flow. This type of reactor has the advantage of low mass-transfer resistance and requires relatively few cells per experiment.

The effects of a glucose pulse on starving cells are shown in Fig. 9, Curve "a." The first peak resulted from the changes in the cytoplasmic NADH pool and the second from changes in the mitochondrial NADH levels. Curve "b" shows the results of injecting cyanide shortly before a glucose pulse: cyanide blocked electron transfer in the respiratory chain, and thus NADH accumulated to a constant level.

In another glucose-pulse experiment, a glucose injection was followed by one containing both glucose and iodoacetate (Fig. 10). The effects of iodoacetate, which inhibits several enzymes in the glycolytic pathway, are clear.

Nutrient Feeding and Metabolic Shifts

Clostridium acetobutylicum is an anaerobic bacterium that can produce a number of products. Fermentations of this organism usually consist of periods characterized as acidogenic (producing chiefly acetic and butyric

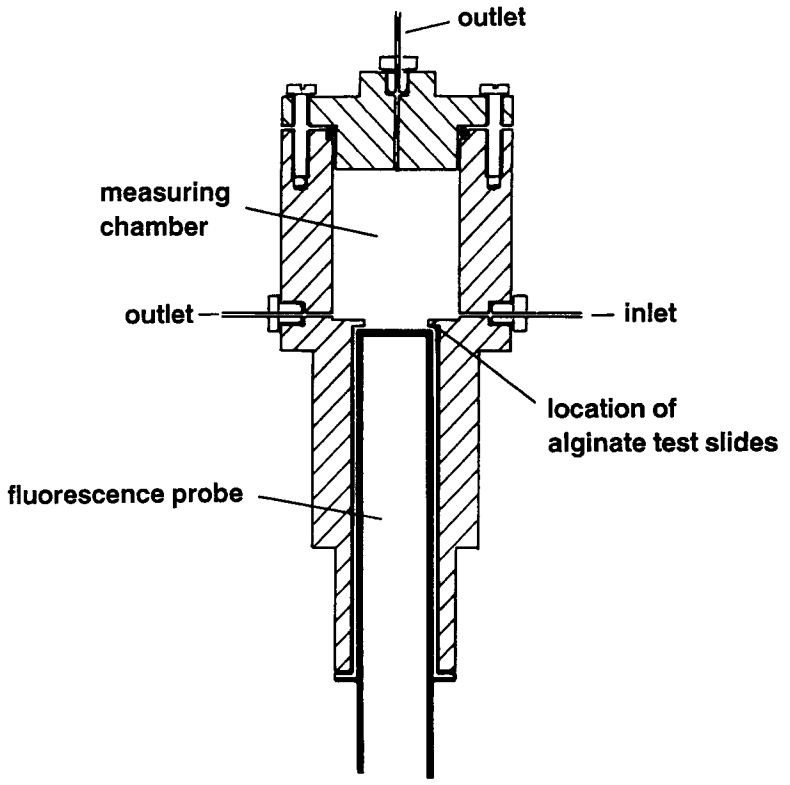

Fig. 8. Flow-through fluorescence cell for pulse experiments. The measuring chamber fits over the tip of the fluorescence probe.

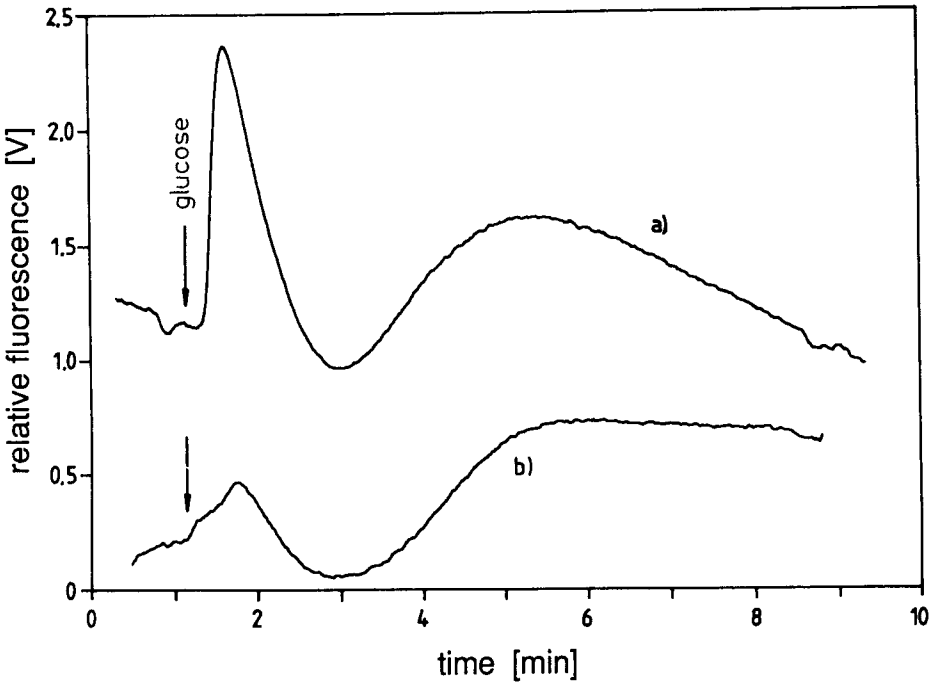

Fig. 9. Glucose-pulse experiments (curves are offset): a) glucose only; b) cyanide, then glucose.

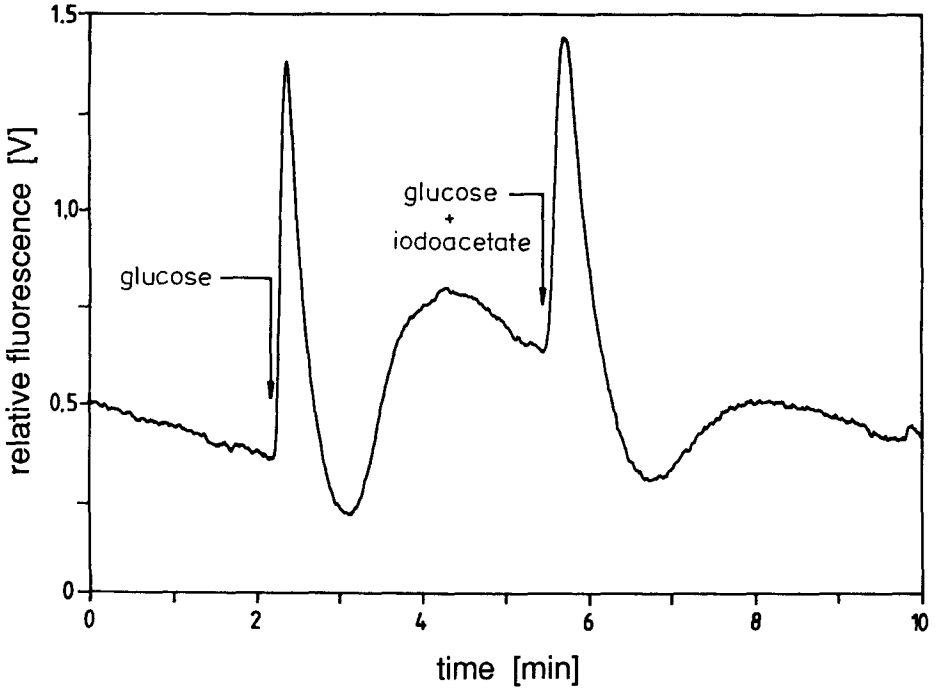

Fig. 10. Glucose pulse followed by glucose/iodoacetate pulse.

acids) or solventogenic (producing mainly acetone, butanol, and ethanol). NAD(P)H-dependent fluorescence was monitored on-line in an investigation on immobilized *C. acetobutylicum* deactivation and regeneration. In this experiment, calcium alginate-immobilized cells were placed in the quartz column of a reactor system similar to that shown in Fig. 5 (adapted for anaerobic work). The Fluorosensor was interfaced to this column at a quartz window. Additional information on this reactor system and the media used can be found in another report (*10*).

After batch growth on a complex nutrient medium the alginate beads were rinsed thoroughly and the system was operated with a continuous flow of a nitrogen-free minimal medium containing only glucose and mineral salts. The time profiles of the glucose and product concentrations, along with the fluorescence trajectory, are shown in Fig. 11; time zero represents the start of continuous medium flow. The dashed line below the fluorescence curve is the cell-free medium background fluorescence, which was low and constant throughout the experiment. During the first (deactivation) phase, the product formation changed from solventogenic to acidogenic, and the NAD(P)H-dependent fluorescence signal reflected this metabolic shift; the observed fluorescence changes can be explained in terms of the metabolic pathways of this microorganism (*8,10*).

At the time marked by the vertical dotted lines, NH_4Cl was injected into the system and added to the medium to begin the activity regeneration phase. Nitrogen was immediately consumed by the cells, glucose

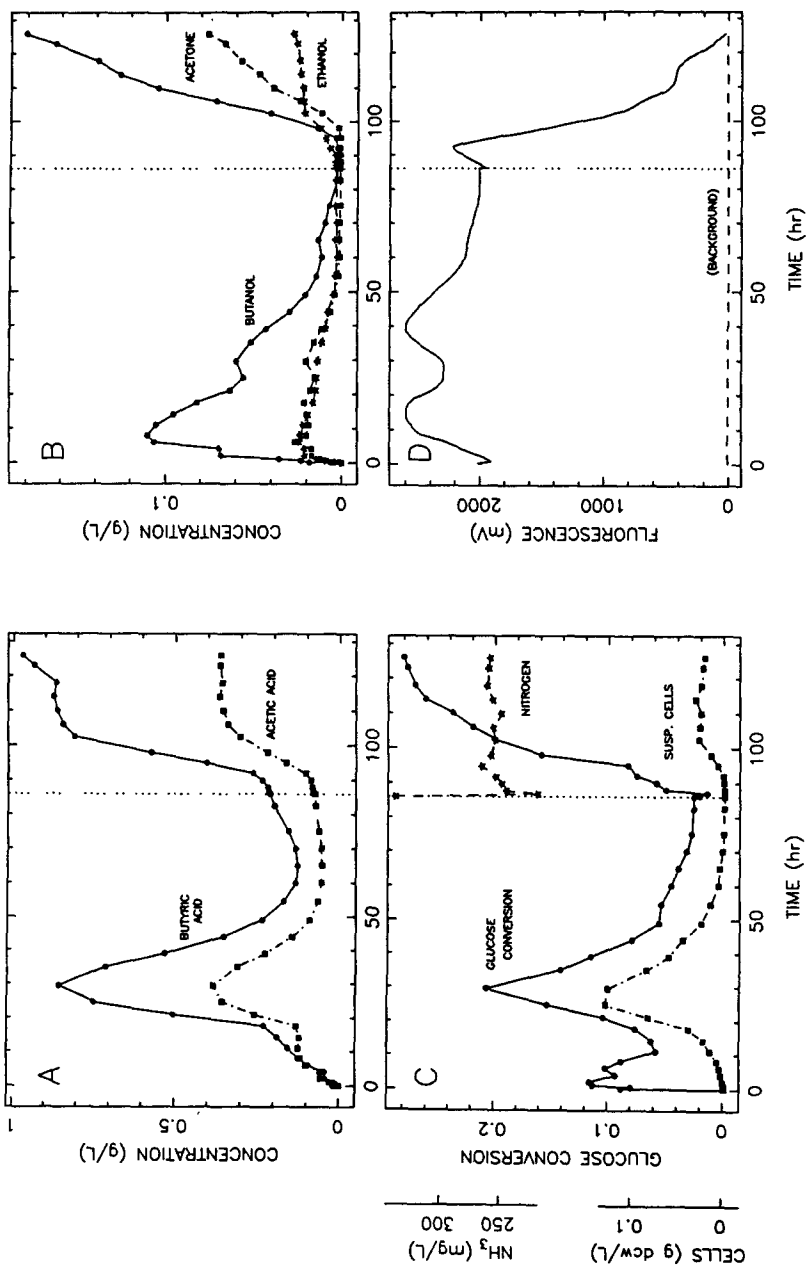

Fig. 11. Results of immobilized *C. acetobutylicum* deactivation and regeneration phases. NH_4Cl addition (the start of the regeneration phase) is marked by the dotted vertical lines. A) Acetic and butyric acids; B) Acetone, butanol, and ethanol; C) Glucose conversion, nitrogen concentration (as ammonia), and suspended cells; D) Fluorescence.

conversion soon began to increase rapidly, and product formation rates increased as well. The fluorescence levels changed significantly during this time, indicating major changes in the redox state of the immobilized cells.

CONCLUSIONS

The applications described here demonstrate the wide range of possible uses of fluorescence probes in bioprocess design and monitoring. Although fluorescence data are not always easy to interpret, the ubiquitous nature of NAD(P)H reactions in living systems, combined with the fact that no other type of sensor is presently available for the on-line, *in situ* bioreactor monitoring of an intracellular characteristic, makes the use of these probes quite attractive.

ACKNOWLEDGMENTS

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REFERENCES

1. Zabriskie, D. W. and Humphrey, A. E. (1978), *Appl. Environ. Microbiol.* **35,** 337–343.
2. Beyeler, W., Einsele, A., and Fiechter, A. (1981), *Eur. J. Appl. Microbiol. Biotechnol.* **13,** 10–14.
3. Scheper, T., Lorenz, T., Schmidt, W., and Schügerl, K. (1987), *Ann. NY Acad. Sci.* **506,** 431–445.
4. Scheper, T., and Bückmann, A. F. (1990), *Biosensors and Bioelectronics.*
5. Bückmann, A. F. (1987), *Biocatalysis* **1,** 173–186.
6. Müller, W., Wehnert, G., and Scheper, T. (1988), *Anal. Chim. Acta* **213,** 47–53.
7. Arminger, W. B., Forro, J. F., Montalvo, L. M., and Lee, J. F. (1986), *Chem. Eng. Commun.* **45,** 197–206.
8. Reardon, K. F., Scheper, T., and Bailey, J. E. (1987), *Biotechnol. Progress* **3,** 153–167.
9. Anders, K.-D., Müller, W., Scheper, T., and Bückmann, A. F. (1989), *Ann. NY Acad. Sci.*
10. Reardon, K. F., Scheper, T., and Bailey, J. E. (1986), *Biotechnol. Lett.* **8,** 817–822.